general

Cancer preventive properties of varieties of *Brassica oleracea:* a review[1-3]

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ABSTRACT Cabbage, broccoli, Brussels sprouts, and other members of the genus *Brassica* have been widely regarded as potentially cancer preventative. This view is often based on both experimental testing of crude extracts and epidemiological data. The experimental evidence that provides support for this possibility is reviewed for the commonly consumed varieties of *Brassica oleracea.* In a majority of cases the biological activities seen in testing crude extracts may be directly related to specific chemicals that have been reported to be isolated from one of these closely related species, thus the chemical evidence further supports the data from testing extracts and epidemiology. *Am J Clin Nutr* 1994;59(suppl):1166S-70S.

KEY WORDS *Brassica,* Brassicaceae, mutagen, antimutagen, cancer, prevention, vegetables, chemoprevention

Introduction

Although most botanists would hardly agree that "A rose may be a rose by any other name" there would be substantial agreement that a cabbage and a cauliflower may be quite the same. These vegetables, and other closely related members of the Brassicaceae family, have received widespread notice recently as public figures have disavowed their consumption and scientists have upheld them as exemplary of medicinaly significant foods. Thus, in this article we review all of the experimental evidence that suggests that there may be a cancer preventive benefit from consumption of members of these closely related and commonly consumed vegetables (1, 2). Furthermore, in view of the extensive data (3, 4) that exist for these vegetables, we will restrict ourselves to those vegetables commonly classified as subvarieties of the species *Brassica oleracea* (**Table 1**).

From the outset it must be realized that the published experimental data come from two different types of experimental protocols. In the first type, evidence is published that concerns tests conducted on the whole food (or from crude extracts). In the second type, tests are conducted on specific chemical compounds that have been isolated from these foods. Specifically, we will cross-correlate these two bodies of data so that, whenever possible, the specific compounds that may be responsible for an observation seen in testing a crude extract are identified. It is worth noting that this information is often not available in the original article and lends credence to the initial observation.

It is our intention to provide support for observations made on crude extract and identify those areas in which the biologically active chemical species for a given observation may not yet be identified. Although various aspects of the chemistry (5), pharmacology (6, 7), biology (8-10), and general concepts of cancer chemoprevention (11-13) have been reviewed separately, we will provide an overview approach that demonstrates the overlap between these various areas. Furthermore, it is important to recognize that many clinical trials are currently underway, (14) which, in preliminary reports, lend credence to the cancer preventative approaches (15, 16).

Relevant biological activities

The etiology of cancer follows no single track but rather is the result of an accumulation of diverse events that lead to a common endpoint, namely the uncontrolled growth of a normally quiescent cell. Nevertheless, there are generally recognized to be many common stages to the development of cancer. These stages (Fig 1) include an initial insult (or mutation) to the genetic material often delivered by a mutagen or other chemical agent but may also be inherited or possibly viral in origin. A cell that has received such an insult is said to be initiated. An initiated cell will still be quiescent and not manifest its altered phenotype until it is promoted. The promotional act may similarly take multiple forms but it fundamentally involves achieving a physiological state that signals the altered DNA to be read. Where the altered message leads to an unquenchable cycle of cellular division, the cell is considered cancerous. This aberrant equilibrium, where the cell cannot reset itself, will become a tumor if it cannot regain a "normal" or self-restrained equilibrium.

Cancer chemotherapeutic agents are directed against cancerous or fully promoted cells and seek to selectively kill the cell based on some aspect of its aberrant biochemical equilibrium. As such, all current cancer treatment is based on compounds that are toxic. An ideal cancer chemotherapeutic agent would be toxic only to cancer cells but the reality is that such specificity has not yet been acheived. Although this is clearly a suitable course when the fatality of the disease is considered, the approach to cancer

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Exhibit 2-G(i)

TABLE 1
The most commonly consumed members of the genus *Brassica*

Species	Variety	Common name
Brassica campestris		Field mustard
Brassica chinensis		Bok choy
Brassica juncea		Mustard greens
Brassica napus	var *napabrassica*	Rutabaga
Brassica nigra		Black mustard
Brassica oleracea	var *acephala*	Collards
Brassica oleracea	var *acephala*	Kale
Brassica oleracea	var *botrytis*	Broccoli
Brassica oleracea	var *botrytis*	Cauliflower
Brassica oleracea	var *capitata*	Cabbage
Brassica oleracea	var *gemmifera*	Brussels sprouts
Brassica oleracea	var *gorgylodes*	Kohlrabi
Brassica pekinensis	var *capitata*	Cabbage (Chinese)
Brassica rapa	var *rapifera*	Turnip
Brassica rapa	var *japonica*	Red turnip

chemoprevention must be based on a very different strategy. In view of the fact that such agents must be used prophylactically, they must exhibit few, if any, side effects and must have virtually no toxicity. In addition to these stringent requirements, it needs to be recognized that any compound that is to be considered as a cancer chemopreventive agent may also exhibit a suitable spectrum of biological activity.

In cancer chemoprevention the aim is to reduce the number of initiated cells, inhibit the promotion of initiated cells, or even reverse the promotion itself. Furthermore, each of these broad categories has many strategies that may be useful to cancer prevention. First, there are strategies that aim to reduce the initiation rate. The agents here are classified as antimutagens, desmutagens, inhibitors of enzymes that activate procarcinogens, or agents that stimulate the metabolization of mutagens to less harmful metabolites. Also included here are antioxidants because a portion of the genetic damage is likely the result of free radical damage (17–21). Second, there are strategies that aim to reverse or inhibit the promotion stages. Biological activities that act at this stage may act specifically on the promoted cell to cause it to redifferentiate and hence regain control of its own division or they may act at any of the points in one of the secondary messenger (or the related oncogene) systems that are frequently implicated as destabilizing agents. In the same light, the biological consequences of low-level inflammation or constant low-level estrogenic stimulation are similarly considered destabilizing (22, 23) and hence targets for chemopreventive approaches.

As we consider such an etiology, we can associate specific bioassays that have been described in the literature in relation to one or more of these points. Thus, as a basis for this article, we have undertaken to review the reports of relevant biological activities for *Brassica oleracea* varieties. They will be organized as discussed above. With respect to initiation stages, the majority of published literature in this area may be divided crudely into two broad groupings, namely reports of an anti- (or des-) mutagenic activity and reports of stimulation of a detoxification mechanism. With respect to antipromotion activity, there is a single report that suggests that this mechanism may play a role in *Brassica's* cancer preventive potential. However, there are many reports that may not be classified as either of these stages but

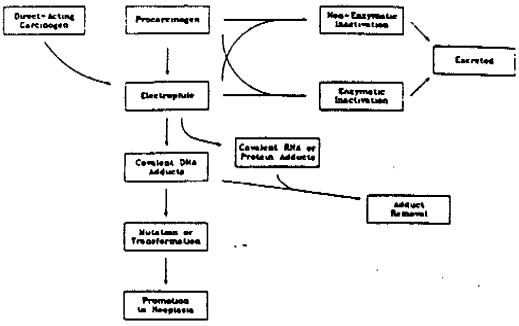

FIG 1. The etiology of cancer. The steps shown are those generally assumed in the development of cancer.

rather are of such a general nature that no specific mechanism of protection may be ascribed to them.

Antimutagenic activities

The ability of a crude extract of a *Brassica* variety to reduce the effect of a mutagen (either as a desmutagenic agent or as an antimutagenic agent) has been reported no less than eight times. In all of the cases in which a mechanism can be discerned it appears that, although the term antimutagen is used routinely, these are most likely all cases of desmutagenicity. These reports are summarized in Table 2.

The major bulk of the reports concern ability of a protein, termed the desmutagenic factor, to inhibit various mutagens in Ames-type assays. This factor, first described by Kada *et al* (24), was later characterized (25) and patented (26) by Morita et al as a heat-labile protein with a molecular weight of ≈53 kDa, which contained a prosthetic group with a heme-like chromophore. This protein was shown active against tryptophan pyrolysates (24), ethidium bromide (25), 2-aminoanthracene (25), autooxidized linolenic acid (27), and pyrolysates for other amino acids (28).

TABLE 2
Summary of antimutagenic results

Plant extracted	Mutagen	Percent reduction
		%
Cauliflower	Nitrate + methylurea	78
Cauliflower	Nitrate + aminopyrine	57
Cabbage	Nitrate + sorbic acid	Moderate (not calculable)
Cauliflower	Nitrate + sorbic acid	Moderate (not calculable)
Cabbage	Tryptophan pyrolysate	97
Broccoli	Tryptophan pyrolysate-1	97
Broccoli	Tryptophan pyrolysate-2	81
Broccoli	Ethidium bromide	92
Broccoli	2-Aminoanthracene	84
Broccoli	AF-2	0
Broccoli	Oxidized linolenic acid	82
Cabbage	Oxidized linolenic acid'	76
Red cabbage	Oxidized linolenic acid	81
Cauliflower	Oxidized linolenic acid	76
Cabbage	Tryptophan pyrolysate-2	35

In their 1980 paper, Yamaguchi et al (27) demonstrated a striking correlation between the desmutagenic activity of the extracts and their peroxidase activity and further demonstrated that the peroxidase activity required a cofactor. This activity was later confirmed in the purified protein by Morita et al (25), who did not note the need for the cofactor. The signal characteristic to the desmutagenic factor has always been the fact that it is both heat labile and is inactivated by digestion with a proteinase. With this in mind, some workers (29) have pointed out that after heat treatment some crude extracts of *Brassica* extracts still exhibit residual activity, suggesting the presence of other antimutagenic components. Munzer (30) demonstrates that some antimutagenic activity acts by stimulating native detoxification systems in *Salmonella typhimurium* and thus some of these other agents are also desmutagenic.

The identity of the other antimutagenic agents has been the focus of other researchers. Two groups (31, 32) have reported that extracts of cauliflower and cabbage, respectively, interfere with the production of mutagens by nitrosation. There is considerable agreement that the active agents include ascorbic acid, cysteine, or other compounds acting as reducing agents. This is actually demonstrated by Osawa et al (32), who show that the ascorbic acid is responsible for the chemical reduction of the 1,2-dinitro-2-methyl pyrrole, the mutagenic nitrosation product of sorbic acid, to the nonmutagenic compound 1-nitro-2-methyl-4-amino pyrrole. Barale et al (31) show that ascorbic acid and some phenolic compounds can duplicate the activity seen in the crude extract. On the other hand, Lawson et al (33) have identified four specific compounds isolated from savoy chieftain cabbage that demonstrated antimutagenic activity against specific mutagens, *N*-methyl-*N*-nitrosourea (NMU) and 2-aminoanthracene (2-AA). These compounds, β-sitosterol, pheophytin-a, nonacosane, and nonacosanone, are notable because they are likely to be present in a majority of plants. These authors also demonstrate that commercial chlorophyll, the biological precursor to pheophytin-a, is strongly antimutagenic. These compounds were shown to present different activity profiles against the NMU and 2-AA; therefore, the authors argue that these compounds were achieving their antimutagenicity through more than one biological mechanism.

Stimulation of detoxification mechanisms

As noted briefly above, Munzer (30) noted that the antimutagenic activity of many vegetables, including cabbage, Brussels sprouts, and kohlrabi, was in stimulating the S-9 mix normally used to metabolize and sometimes activate mutagens. This observation serves to bridge the antimutagenic potential discussed above and the large body of data that makes it clear that in animals there is a strong stimulation of many of the native detoxification systems by extracts of various *Brassica* species. Although this attribute has been fairly widely discussed recently, because of the articles published by Talalay's group (34, 35), it is important to note that this area has a long and honorable background. Furthermore, although the Talalay articles do demonstrate a selectivity in the induction of phase-2 enzymes that has not previously reported, the ability of members of the Brassicaceae family to stimulate a broad spectrum of enzyme systems has been widely reported.

The earliest work on the induction of these enzyme systems was actually an attempt by Wattenberg (36) to explain variations in baseline aryl hydrocarbon hydroxylase concentrations in different rat colonies. The variation ultimately was ascribed to the presence of alfalfa as an occasional component in rat chow. This observation was followed by an examination of the ability of many foods to stimulate this enzyme. Wattenberg and his group demonstrated that many members of the Brassicaceae family were also active in this regard (37) and, furthermore, the active compounds were readily identified as indole-3-carbinol, 3,3'-diindolymethane, and indole-3-acetonitrile, which stimulated 50-fold, 20-fold, and 6-fold increases, respectively, in enzyme activities in the livers of rats that consumed augmented basal chow. In subsequent papers they demonstrated that the ability of intestinal enzymes to detoxify many xenobiotic compounds, including the indoles noted above (38), correlated to Brussels sprouts or cabbage consumption in rats (39) and in humans (40). The enzyme systems involved included many mixed-function oxidases, such as phenacetin O-dealykylase, 7-ethoxycoumarin O-dealkylase, hexobarbital hydroxylase, and benzo[a]pyrene hydroxylase. A direct correlation was later established between the induction of these activities and the concentration of these compounds by McDanell et al (41, 42). These later studies also demonstrated that the various active compounds had differing abilities to stimulate enzymes in different organs of the body. They note for instance that the ascorbic acid conjugate of indole-3-carbinol is the most active compound in stimulating the mixed-function oxidase populations of the gut whereas indole-3-carbinol, of the compounds tested, was the strongest inducer of the liver enzymes. Tanaka et al (43) demonstrated recently the ability of indole-3-carbinol to inhibit tongue carcinogenesis induced with 4-nitroquinoline-1-oxide.

Meanwhile, working in a parallel vein, Salbe and Bjeldanes (44) not only confirmed the earlier results of the Wattenberg group but also demonstrated that the enzyme glutathione-*S*-transferase was also strongly induced by Brussels sprouts. This enzyme, unlike those discussed earlier, is not a P-450 type enzyme but represents rather a phase-2 detoxification system that acts to conjugate and clear toxicants from the system. The significance of this difference cannot be understated. For most of the P-450 type enzymes, their ability to detoxify many mutagens must always be balanced by their ability to activate other mutagens (45). For glutathione-*S*-transferase, there are no such drawbacks, rather, as this group has shown (46), an increase in this enzyme alone directly resulted in an 87% reduction in the binding of aflatoxin to hepatic DNA in vivo. A wide spectrum of compounds (47, 48) including the glucosinolates, such as sinigrin and progoitrin, and their derivates, such as allyl isothiocyanate, goitrin, indole-3-carbinol, and indole-acetonitrile, induce glutathione-*S*-transferase. In other systems it is induced even more strongly by xanthotoxin and some flavanoids (49).

Other relevant reports

There are some reports in which no mechanism can be easily ascribed to the results or that do not fit into either of the above two categories. These reports are nonetheless potentially significant with respect to the ability of brassicaceous plants to be cancer chemopreventive. The first of these concerns a study conducted by Bresnick et al (50) in which rats were fed a controlled fat diet with and without cabbage. There was found to be a statistically significant reduction in the rate of chemically induced

breast tumors in the rats with cabbage in their diet. This effect was not seen in rats on a high-fat diet. It is of interest to note that the experimental design allowed for the consumption of cabbage only after the initiation event, administration of MNU, thus indicating a potential antipromotion effect. This possibility is also suggested by a report from Koshimizu et al (51), who use the inhibition of Epstein-Barr virus induction as an indication of antipromotion activity. In their assay an extract of cauliflower is very strongly active in inhibiting the normal promotion event. In neither of these publications is it possible to ascribe a specific compound to the activity observed.

Finally, note that protease inhibitors have been associated with carcinogenesis inhibition (52, 53), so the relevance of a strong trypsin inhibitor from the seed of kale (*Brassica oleracea* var *sabellica*) may be relevant (54). The presence of this agent in other parts of the plant (much less in other varieties) or its ability to overcome problems of absorption and transport are totally unknown.

Summary

It may at first seem surprising that so many biological activities have been demonstrated for plants as commonly consumed as these. Yet reflection on the complex chemical nature of most plants suggests that there may be more biological potential in all of them than we would expect from something that is generally considered to be biologically neutral. Furthermore, although some of these reports have been in humans, the majority are in vitro results whose bearing on their effect on humans is very much an open question. The work of McDanell et al (41, 42) clearly demonstrates the importance of transport and the variable ability of different metabolites of even the same compound to affect different organs. A report by Birt et al (55) amplifies this by demonstrating that although the effect of cabbage is beneficial in some cases it may act to increase tumorigenicity in other model systems (or cancer types).

We have presented a case that strongly implies that the cancer preventive potential of many members of the Brassicaceae family is strong, yet it must always be stressed that to understand the relevance of these reports on the human condition, many further studies need to be done to specifically address questions of the stability, bioavailability, transport, and metabolism. The additive or even synergistic effects of these compounds are unknown. The additional effects of normal food preparation procedures present another factor that is yet largely unexplored with respect to the cancer preventive properties. In brief, there is much exciting potential in the cancer preventive properties and yet there is, as of this writing, no absolute statement that can be made concerning the ability of these foods to directly alter the course of carcinogenesis.

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Chemical and molecular regulation of enzymes that detoxify carcinogens

(chemoprotection/electrophiles/quinone reductase/transient gene expression/phase 2 enzymes)

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ABSTRACT **Inductions of detoxication (phase 2) enzymes, such as glutathione transferases and NAD(P)H:(quinone-acceptor) oxidoreductase, are a major mechanism for protecting animals and their cells against the toxic and neoplastic effects of carcinogens. These inductions result from enhanced transcription, and they are evoked by diverse chemical agents: oxidizable diphenols and phenylenediamines; Michael reaction acceptors; organic isothiocyanates; other electrophiles—e.g., alkyl and aryl halides; metal ions—e.g., $HgCl_2$ and $CdCl_2$; trivalent arsenic derivatives; vicinal dimercaptans; organic hydroperoxides and hydrogen peroxide; and 1,2-dithiole-3-thiones. The molecular mechanisms of these inductions were analyzed with the help of a construct containing a 41-bp enhancer element derived from the 5′ upstream region of the mouse liver glutathione transferase Ya subunit gene ligated to the 5′ end of the isolated promoter region of this gene, and inserted into a plasmid containing a human growth hormone reporter gene. When this construct was transfected into Hep G2 human hepatoma cells, the concentrations of 28 compounds (from the above classes) required to double growth hormone production, and the concentrations required to double quinone reductase specific activities in Hepa 1c1c7 cells, spanned a range of four orders of magnitude but were closely linearly correlated. Six compounds tested were inactive in both systems. A 26-bp subregion of the above enhancer oligonucleotide (containing the two tandem "AP-1-like" sites but lacking the preceding ETS protein binding sequence) was considerably less responsive to the same inducers. We conclude that the 41-bp enhancer element mediates most, if not all, of the phase 2 enzyme inducer activity of all of these widely different classes of compounds.**

Elevation of the activities of phase 2 detoxication enzymes† of cells provides protection against neoplasia (6). This paper analyzes the chemical and molecular specificity of the regulation of phase 2 enzymes, as part of our efforts to develop novel approaches to chemoprotection against cancer. Phase 2 enzymes, which are widely distributed in mammalian cells and tissues, include the following: glutathione (GSH) transferases, which conjugate mostly hydrophobic electrophiles with GSH; QR, which promotes obligatory two-electron reductions of quinones, preventing their participation in oxidative cycling and the depletion of intracellular GSH; epoxide hydrolase, which inactivates epoxides and arene oxides by hydration to diols; and UDP-glucuronosyltransferases, which conjugate xenobiotics with glucuronic acid, thus facilitating their excretion. The induction of these enzymes is accompanied by elevations of intracellular GSH levels which augment cellular protection (7–11).

Induction of phase 2 enzymes is evoked by an extraordinary variety of chemical agents, including Michael reaction acceptors, diphenols, quinones, isothiocyanates, peroxides, vicinal dimercaptans, heavy metals, arsenicals, and others (12–14). With few exceptions these agents are electrophiles (or can be converted to electrophiles by metabolism), and accordingly, many of these inducers are substrates for glutathione transferases (13).

The molecular basis of the regulation of phase 2 enzyme inductions has been analyzed by deletions of the 5′ upstream regulatory regions of glutathione transferase Ya subunit genes and QR genes after transfection of cells with chloramphenicol acetyltransferase (CAT) constructs (3, 15–17). The sequences of the upstream enhancer elements of the mouse and rat liver glutathione transferase Ya subunit genes that respond to the few inducers tested are very similar and have been termed the electrophile-responsive element (EpRE) (18) and the antioxidant-responsive element (ARE) (19), respectively. These elements (Fig. 1) are contained within a 41-nt segment located between base pairs -754 and -714 in the mouse, and -722 and -682 in the rat Ya gene. The critical DNA sequences responsive to monofunctional inducers appear to be the TGACAT/AT/AGC regions, which resemble AP-1 binding sites (20). Similar enhancer sequences have also been identified in the upstream regulatory regions of the rat and human QR genes (3, 4, 17). We show that all the chemical inducers of phase 2 enzymes that we tested stimulate expression of a reporter gene through this 41-bp enhancer element.

MATERIALS AND METHODS

Cell Culture. For the growth hormone (GH) transient gene expression assays the cells were grown in Eagle's minimal

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Abbreviations and definitions: AP-1, a family of transcriptional activator DNA-binding proteins that bind to the consensus sequence TGAC/GTC/AA; CAT, chloramphenicol acetyltransferase; CD_{GH}, concentration of an inducer that doubles the production of growth hormone in a transient gene expression assay; CD_{QR}, concentration of an inducer that doubles the quinone reductase specific activity in Hepa 1c1c7 cells; DMSO, dimethyl sulfoxide; ETS, a family of transcriptional activator DNA-binding proteins; GH, growth hormone; QR, quinone reductase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2]; sulforaphane, 1-isothiocyanato-(4*R*)-(methylsulfinyl)butane [CH_3—SO—$(CH_2)_4$—NCS].

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†Two broad classes of enzymes metabolize xenobiotics: (*i*) phase 1 enzymes, which functionalize molecules by introducing hydroxyl or epoxide groups and (*ii*) phase 2 enzymes (1), which detoxify either by conjugating these functionalized molecules with endogenous ligands (e.g., glutathione), thus facilitating their excretion, or by destroying their reactive centers by other reactions [e.g., hydrolysis of epoxides by epoxide hydrolase or reduction of quinones by quinone reductase (QR)]. Reasons for considering QR a phase 2 enzyme are presented elsewhere (2–4). Inducers of enzymes of xenobiotic metabolism belong to two families (5): (*i*) bifunctional inducers, which bind to the aryl hydrocarbon (Ah) receptor and induce certain phase 1 enzymes and phase 2 enzymes and (*ii*) monofunctional inducers, which induce phase 2 enzymes independently of the Ah receptor.

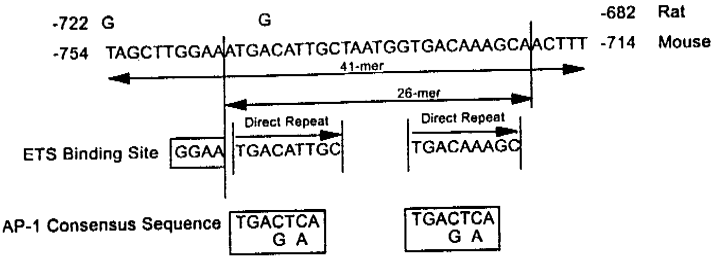


FIG. 1. Highly homologous 41-bp enhancer sequences from the upstream region of the mouse and rat glutathione transferase Ya subunit genes, representing bases -682 to -722 (rat) and -714 to -754 (mouse) from the origins of replication. Two "AP-1-like" regions are present with a core ETS protein binding site located next to the first AP-1 site in the mouse sequence.

essential medium supplemented with Earle's balanced salt solution, nonessential amino acids, sodium pyruvate, glutamine, and 10% fetal calf serum. All cells were maintained in a humidified atmosphere of 5–7% CO_2 at 37°C. Cell lines were free from mycoplasma.

Compounds. Most inducers were obtained commercially. Racemic sulforaphane was synthesized by C.-G. Cho and G. H. Posner (21).

Plasmids and Their Constructions. The plasmids 41YaCAT and -187YaCAT (15) and RSVgal were gifts of Violet Daniel (The Weizmann Institute of Science, Rehovot, Israel). The plasmid pCH110 was obtained from Pharmacia. p41YaCAT contains a portion of the upstream sequence of the mouse glutathione transferase Ya gene from -1594 to the *Bgl* II site at -1272 where the 41-bp EpRE is present in the reverse orientation (20). The EpRE is linked directly to the intact *Bgl* II site at nucleotide -187 in the upstream region. p284YaGH was prepared by ligating a 284-nt fragment (representing the sequence from -186 to +98) containing the mouse glutathione transferase Ya minimal promoter (as in -187 YaCAT) region into the *Bam*HI site of plasmid p0GH (22). The 284-nt fragment was generated by PCR from the plasmid 41YaCAT by use of the primers 5′-GGC TTC ACT CCA TCT AGA AAG GG-3′ and 5′-TTG CAG TGC TGC AGA CCT GGG AA-3′. The fragment was gel purified, its ends were blunted, and *Bam*HI linkers were added. It was then digested with *Bam*HI and *Bgl* II to generate two fragments, the smaller, 284-nt, fragment containing 186 nucleotides of the upstream region, the first exon, and 56 nucleotides of the first intron of the mouse glutathione transferase Ya gene. The plasmid p26-284GH was prepared by first ligating the oligonucleotide 5′-agc ttA TGA CAT TGC TAA TGG TGA CAA AGC Ag-3′ (lowercase letters indicating restriction overhangs) and its complement 5′-gat ccT GCT TTG TCA CCA TTA GCA ATG TCA Ta-3′ into p0GH (which had been cleaved with *Hin*dIII and *Bam*HI) to provide p26GH. The 284-nt fragment containing the glutathione transferase Ya minimal promoter was then inserted into the *Bam*HI site of the plasmid p26GH. The plasmid p41-284GH was prepared by ligating the oligonucleotide 5′-agc tTA GCT TGG AAA TGA CAT TGC TAA TGG TGA CAA AGC AAC TTT g-3′ and its complementary oligonucleotide 5′-tcg acA AAG TTG CTT TGT CAC CAT TAG CAA TGT CAT TTC CAA GCT A-3′ into the *Hin*dIII and *Sal* I sites of p284GH. The structures of all DNA constructs were confirmed by automated sequencing.

Transfections and Transient Gene Expression Assays. Transfections were performed by the calcium phosphate method (23). Briefly, the cells were plated at a density of 3.5×10^6 (Hepa 1c1c7) or 7×10^6 (Hep G2) in 10-cm plates and medium was replaced after 14–16 hr. After a further 3 hr, the transfection mixture containing 20 μg of the specified GH construct and 12 μg of the β-galactosidase construct (RSVgal for Hep G2 and pCH110 for Hepa 1c1c7) was added. Five hours later the cells were shocked with 15% (wt/vol) glycerol for 2 min and then allowed to recover for 16–18 hr. The cells from each 10-cm plate were trypsinized and pooled. One-quarter of the cells from each plate were replated onto a 10-cm dish for β-galactosidase assay, and the remaining cells were distributed among the wells of three 24-well plates containing 1.5 ml of medium per well. After 3–4 hr the cells were treated with three or more concentrations of inducers dissolved in either dimethyl sulfoxide (DMSO) or water (arsenicals and metal salts). A final concentration of 0.2% (vol/vol) DMSO was present in all assays. After a further 48 hr, 100 μl of medium was removed from each duplicate well and assayed for GH (Allégro HGH Transient Gene Expression Assay Kit; Nichols Institute, San Juan Capistrano, CA). CAT and β-galactosidase assays were performed (23), and viability was determined by staining with crystal violet (24).

Standardization of GH Gene Expression Assay. Basal GH secretions in six independent transfections with p41-284GH in Hep G2 cells were as follows (ng of GH secreted per ml of medium in 48 hr; means of n replicates ± the coefficient of variation): 2.20 ± 2.3% (n = 6); 2.67 ± 6.9% (n = 6); 2.80 ± 5.2% (n = 6); 3.27 ± 5.4% (n = 5); 4.93 ± 12.5% (n = 4); and 6.54 ± 4% (n = 4). The mean GH production in these six transfections was 3.92 ng/ml with an uncorrected interassay coefficient of variation of ±45.7%; after normalization for transfection efficiency by β-galactosidase measurements and for cell number (by staining with crystal violet), the interassay coefficient of variation decreased to ±17%. Similar results were obtained in transfections of Hepa 1c1c7 cells. Before transfection, neither Hep G2 nor Hepa 1c1c7 cells expressed detectable GH. Furthermore, GH added to the assay systems (0–10 ng/ml; n = 6) was recovered quantitatively from the medium. GH addition did not alter the expression of GH by Hep G2 cells transfected with p41-284GH.

Comparison of Human GH and CAT Measurements. DNA elements involved in transcriptional regulation of phase 2 enzymes were previously identified by use of constructs containing the CAT reporter gene (3, 4, 15–17). To perform large numbers of assays rapidly and reproducibly, we chose the human GH gene as reporter (22) because the GH radioimmunoassay is simple, extremely sensitive, and highly quantitative. Transfection of cells in a single 10-cm culture plate permits more than 100 measurements and avoids problems associated with differences in transfection efficiencies. To verify that GH and CAT assays gave directly comparable results with our specific enhancer elements, we showed that GH and the CAT assays performed in both Hepa 1c1c7 and Hep G2 cells transfected with the p41YaCAT and p41-284GH plasmids and treated with 2,3-dimercapto-1-propanol gave parallel inductions (Table 1). In both cell lines, however, the GH assay was much more sensitive and the inductive response range (expressed as treated-to-untreated ratios) was much higher in the GH assay than in the CAT assay. Similar response patterns were observed with several other chemically unrelated inducers such as sulforaphane (data not shown). The expression of GH by Hepa 1c1c7 and Hep G2 cells transfected with the enhancerless but promoter-containing plasmids (187YaCAT and p284GH) was not increased by any of the inducers.

Table 1. Responses to 2,3-dimercapto-1-propanol of CAT and human GH transient gene expression assays in Hepa 1c1c7 and Hep G2 cells

	Response ratio (treated/untreated) of cells			
	CAT		GH	
2,3-Dimercapto-1-propanol, μM	Hepa 1c1c7	Hep G2	Hepa 1c1c7	Hep G2
25	1.6	1.3	2.8	2.7
50	2.5	2.6	5.0	6.9
100	3.5	4.7	6.9	18.5

The cells were transfected with the CAT reporter p41YaCAT or the GH reporter p41-284GH.

We conclude that the GH transient gene expression assay in Hep G2 cells is a highly sensitive, quantitative, and reproducible measure of transcriptional regulation and that the results obtained parallel those of CAT assays.

Measurement of Potency for Induction of QR. The inducer potency of all compounds was determined with Hepa 1c1c7 cells grown in 96-well microtiter plates (24, 25). The inducers were added in either DMSO or water. A final concentration of 0.2% DMSO was present in all wells. The CD_{QR} (concentration required to double QR specific activity) values shown in Table 2 are lower than those reported previously (12), probably due to minor modifications [use of fetal calf serum treated with charcoal (1 g/100 ml) for 90 min at 55°C, and the inclusion of 0.2% DMSO in all assays].

RESULTS AND DISCUSSION

Comparison of Efficiencies of Inducer Responses of Plasmids p26-284GH and p41-284GH in Transient Gene Expression Assays. Prior studies of the mouse enhancer sequence used the entire 41-nt segment containing both AP-1-like sites and additional flanking sequences (Fig. 1). To determine whether the two AP-1 sites are sufficient for maximal induction, we compared the expression of GH by the complete construct p41-284GH and by p26-284GH, which contains both of the AP-1-like sites but lacks 10 of the 5′ base pairs and 5 of the 3′ base pairs of the 41-mer (Fig. 1) originally identified to contain the enhancer element in the mouse and rat upstream regions (16, 18, 26, 27). GH expression was measured with a series of concentrations of the following inducers (for structures, see Table 2): 1-nitro-1-cylcohexene (**1**), *trans*-4-phenylbut-3-en-2-one (**10**), *tert*-butylhydroquinone (**14**), sulforaphane (**15**), 2,3-dimercapto-1-propanol (**21**), phenylarsine oxide (**26**), sodium arsenite (**27**), mercuric chloride (**28**), phenylmercuric chloride (**31**), 1,2-dithiole-3-thione (**33**), and β-naphthoflavone (**34**) (Fig. 2).

The basal levels of GH production by both plasmids were essentially identical when corrected for cell number and transfection efficiency. All of these compounds produced concentration-dependent inductions of GH synthesis. Surprisingly, the maximal elevations of GH produced by these compounds in cells transfected with p26-284GH were low compared with experiments with p41-284GH (Fig. 2). However, the results obtained with p26-284GH were comparable to those observed by us (data not shown) and others with similar enhancer sequences in CAT assays (19). Thus, maximal inductions obtained with p26-284GH were 3.5-fold with 60 μM *trans*-4-phenylbut-3-en-2-one and 100 μM 1,2-dithiole-3-thione. The absolute induction ratios obtained with the plasmid containing the larger insert were dramatically higher; the highest induction ratios were 24.6-fold for 60 μM *tert*-butylhydroquinone and 22.5-fold for 6 μM sulforaphane. All compounds tested showed this difference in induction ratios for the two constructs, but the effects with phenylarsine oxide, sodium arsenite, $HgCl_2$, and phenylmercuric chloride were smaller (Fig. 2). There were also large differences in the responses to inducers when p41-284GH and p26-284GH were transfected into Hepa 1c1c7 cells, although the magnitudes of induction ratios in this cell line were somewhat smaller.

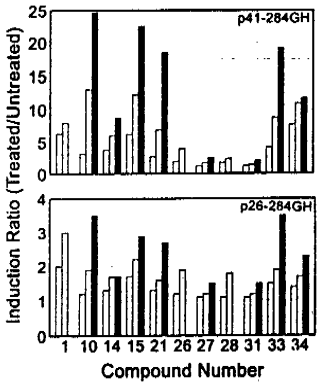


FIG. 2. Effect of different concentrations of inducers on GH production in Hep G2 cells transfected with p41-284GH (*Upper*) or p26-284GH plasmids (*Lower*). The compounds are numbered as in Table 2 and their concentrations (μM) were as follows: **1**, 1-nitro-1-cyclohexene (2.5, 5.0); **10**, *trans*-4-phenylbut-3-en-2-one (20, 40, 60); **14**, *tert*-butylhydroquinone (20, 40, 60); **15**, sulforaphane (1.5, 3.0, 6.0); **21**, 2,3-dimercapto-1-propanol (25, 50, 100); **26**, phenylarsine oxide (0.05, 0.10); **27**, sodium arsenite (2.5, 5.0, 10.0); **28**, mercuric chloride (1.25, 2.5); **31**, phenylmercuric chloride (0.5, 1.0, 2.0); **33**, 1,2-dithiole-3-thione (25, 50, 100); **34**, β-naphthoflavone (0.5, 1.0, 2.0). Open bars, low concentration; shaded bars, double the low concentration; solid bars, high concentration.

In similar experiments with the rat enhancer sequence, Rushmore *et al.* (19) obtained only a 2- to 2.5-fold enhancement of CAT expression. In contrast, Friling *et al.* (18), using the 41-bp mouse enhancer sequence and the same inducers, obtained a 5- to 6-fold elevation in CAT activity, which is in accord with our results (Fig. 2). The responses of the mouse and rat enhancer sequences to inducers may differ because the 5′ region of the mouse 41-bp enhancer contains the core ETS protein DNA-binding sequence GGAA (28) near the first AP-1-like site. Adjacent ETS and AP-1 sites are known to confer dramatic synergism on gene expression (29). The rat gene lacks the first AP-1-like site, because the critical A of the AP-1 consensus is replaced by G (Fig. 1). Whether the

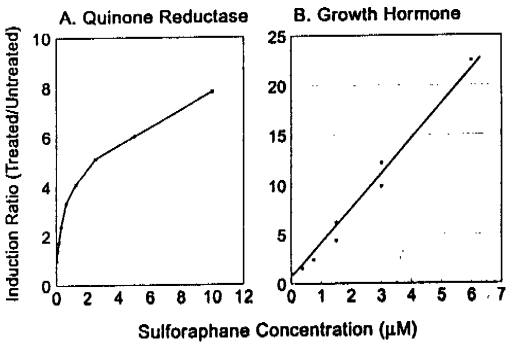


FIG. 3. Effect of increasing concentrations of sulforaphane on QR specific activity (*A*) and GH production (*B*). *B* includes data from two independent transfections, normalized for transfection efficiency.

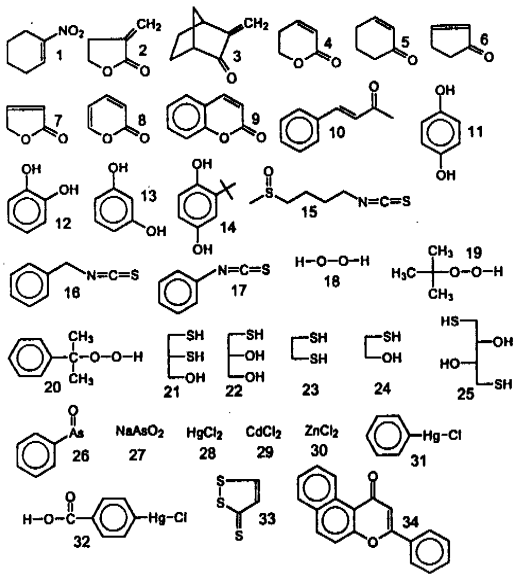
Table 2. Potencies of inducers in enhancing GH production in Hep G2 cells transfected with p41-284GH and in elevating QR activity in Hepa 1c1c7 cells

Inducer No.	Name	CD_{GH}, μM	Rank order	CD_{QR}, μM	Rank order
Michael reaction acceptors					
1	1-Nitro-1-cyclohexene	0.98	5	0.46	5
2	2-Methylene-4-butyrolactone	2.4	8	4.5	12
3	3-Methylene-2-norbornanone	3.1	9	1.5	9
4	5,6-Dihydro-2*H*-pyran-2-one	8.8	12	6.7	16
5	1-Cyclohexen-2-one	15	17	9.1	17
6	1-Cyclopenten-2-one	80	25	32	22
7	2(5*H*)-Furanone	240	28	36	23
8	2*H*-Pyran-2-one	In	29	In	29
9	Coumarin	In	29	In	29
10	*trans*-4-Phenylbut-3-en-2-one	16	20	15	20
Diphenols and quinones					
11	Hydroquinone	12	16	5.3	14
12	Catechol	8.5	11	4.5	12
13	Resorcinol	In	29	In	29
14	*tert*-Butylhydroquinone	11	14	6.0	15
Isothiocyanates					
15	Sulforaphane	0.43	3	0.21	4
16	Benzyl isothiocyanate	0.70	4	3.7	11
17	Phenyl isothiocyanate	In	29	In	29
Peroxides					
18	Hydrogen peroxide	210	27	560	28
19	*tert*-Butyl hydroperoxide	29	23	140	24
20	Cumene hydroperoxide	21	21	210	26
Mercaptans					
21	(±)-2,3-Dimercapto-1-propanol	26	22	12	19
22	3-Mercaptopropane-1,2-diol	In	29	In	29
23	1,2-Ethanedithiol	15	17	21	21
24	2-Mercaptoethanol	180	26	170	25
25	(±)-1,4-Dithiothreitol	In	29	In	29
Trivalent arsenicals					
26	Phenylarsine oxide	0.047	1	0.057	2
27	Sodium arsenite	11	14	2.4	10
Heavy metal salts					
28	$HgCl_2$	1.9	6	0.52	6
29	$CdCl_2$	7.3	10	11	18
30	$ZnCl_2$	73	24	220	27
31	Phenylmercuric chloride	2	7	0.12	3
32	*p*-Chloromercuribenzoate	9.2	13	1.1	8
Other inducers					
33	1,2-Dithiole-3-thione	15	17	1.0	7
34	β-Naphthoflavone	0.051	2	0.029	1

Rank order refers to potencies. When two compounds were equipotent they were assigned the same rank, and the subsequent rank was omitted. Inactive (In) is defined as less than a 20% increase in the induction ratio (treated/untreated) at the highest concentration at which there was less than 50% cell toxicity. *(Structures are shown at top of next column.)*

differences in the inducer response of the mouse and rat enhancers can be attributed to this change requires further mutation and deletion experiments.

Comparison of Potencies of Inducers in Enhancing GH Production in Hep G2 Cells Transfected with p41-284GH and in Elevating QR Activities in Hepa 1c1c7 Cells. To determine whether the transcriptional activation mediated through the 41-bp enhancer element accounted for the entire phase 2 enzyme induction produced by all classes of inducers, we compared the concentrations of inducers required to double GH production (CD_{GH}) and QR activity (CD_{QR}) in the two systems. Typical response curves for sulforaphane (0–10 μM) are shown in Fig. 3*A* (QR induction) and Fig. 3*B* (GH production). Notably, the response ratios at high concentrations of sulforaphane (6 μM) were much higher in the GH assay (22-fold) than in the QR assay (6.4-fold). These graphs generated $CD_{QR} = 0.21 \pm 0.05$ μM and $CD_{GH} = 0.42 \pm 0.18$ μM for sulforaphane (Table 2).



An extraordinary diversity of chemical compounds are active in both systems. Various chemical classes of compounds were tested (Table 2): (*i*) *Michael reaction acceptors* (olefins conjugated to electron-withdrawing functions). As shown for QR induction (12, 13), the potency orders for GH production paralleled the electrophilicity of these compounds. For example, 1-nitro-1-cyclohexene (CD_{GH} = 0.98 μM; CD_{QR} = 0.46 μM), with the olefin conjugated to the powerful electron-withdrawing nitro group, is much more potent in both systems than coumarin (inactive) which is an olefinic lactone. (*ii*) *Diphenols*. Oxidizable diphenols—hydroquinone, catechol, and *tert*-butylhydroquinone—were all comparably potent in both systems, whereas the nonoxidizable resorcinol was inactive (30). (*iii*) *Isothiocyanates*. Sulforaphane was very potent, benzyl isothiocyanate less potent, and phenyl isothiocyanate inactive (12). (*iv*) *Peroxides*. These compounds were all weakly active. Cumene hydroperoxide was slightly more active than *tert*-butyl hydroperoxide, and both compounds were considerably more active than hydrogen peroxide, which induced weakly in both systems (13). (*v*) *Mercaptans*. Mercaptans (which are not electrophiles) were especially active when two thiol groups were adjacent, as in 1,2-ethanedithiol and 2,3-dimercapto-1-propanol (14). 2-Mercaptoethanol was only weakly active, and both dithiothreitol and 3-mercaptopropane-1,2-diol were inactive. Thus two adjacent thiol groups appear to lead to significant inductive potency (14). (*vi*) *Trivalent arsenicals*. Phenylarsine oxide was the most potent inducer tested and was very much more potent than sodium arsenite (14). (*vii*) *Heavy metal salts*. $HgCl_2$, $CdCl_2$, and $ZnCl_2$ were also inducers, with potencies decreasing in this order, which parallels their binding affinity for sulfhydryl groups (14). (*viii*) *Other inducers*. The metabolizable polycyclic aromatic hydrocarbon β-naphthoflavone, a bifunctional inducer, was also a very potent transcriptional activator, doubling the GH production at a concentration of only 0.051 μM. Furthermore, 1,2-dithiole-3-thione also enhanced transcriptional activation through the same enhancer element.

Table 2 shows that 6 of 34 compounds from the eight chemically dissimilar classes were inactive in both systems and none was inactive in only one system. The remaining 28 active inducers ranged in potencies over nearly four orders of magnitude from phenylarsine oxide (CD_{GH} = 0.047 μM; CD_{QR} = 0.057 μM) to hydrogen peroxide (CD_{GH} = 210 μM; CD_{QR} = 560 μM), and many compounds were nearly equipotent in the two assay systems. A plot of potencies of QR induction with respect to potencies of GH production for all active inducers (Table 2) gave a good linear correlation, with an r value of 0.89 and a slope of 0.89 (Fig. 4). We conclude that the induction of QR by all of the very different types of inducers is probably mediated entirely through the 41-bp enhancer element and that GH production and QR induction are controlled by the same or very similar rate-limiting processes. Furthermore, comparison of absolute CD values in the two assays gave a linear correlation (r = 0.64), and the slope of the correlation line was 1.17, indicating that the compounds were nearly equipotent in the two assays.

Conclusions. We have demonstrated that a 41-bp enhancer element from the 5′ upstream region of the mouse glutathione transferase Ya gene (20) is responsive to a wide variety of xenobiotic compounds that also induce phase 2 detoxication enzymes in cultured cells and in animals. Transcriptional activation through this element accounts for most, if not all, of the enzyme elevations produced by these inducers. The inducers belong to many different chemical classes; most contain electron-deficient centers and their potencies parallel the strengths of the electron-withdrawing functions. Furthermore, inducers are also substrates for glutathione transferases, thus emphasizing their electrophilicity (13). Paradoxically, dimercaptans were also found to be inducers. The only apparently universal property of all inducers is their capacity for reaction with sulfhydryl groups (by oxidoreduction or alkylation). We suggest, therefore, that a mechanism involving protein thiol modifications modulates the transcriptional activations mediated by the 41-bp enhancer element. In this connection, it is of considerable interest that the redox state of sulfhydryl groups has been implicated in AP-1 binding to DNA (31–33).

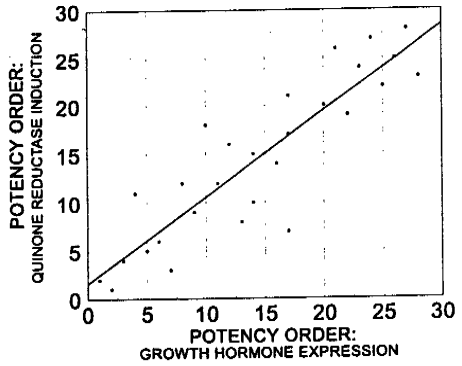


FIG. 4. Order of potencies of 28 compounds in inducing QR (CD_{QR}) and in stimulating growth hormone production (CD_{GH}). The 28 active compounds (Table 2) were ranked from 1 to 28 in order of their potencies in the QR (ordinate) and GH (abscissa) assays. Inactive compounds were excluded. There is a good linear correlation (r = 0.89 and slope = 0.89).

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Anticarcinogenic Activities of Organic Isothiocyanates: Chemistry and Mechanisms[1]

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Abstract

Organic isothiocyanates block the production of tumors induced in rodents by diverse carcinogens (polycyclic aromatic hydrocarbons, azo dyes, ethionine, *N*-2-fluorenylacetamide, and nitrosamines). Protection is afforded by α-naphthyl-, β-naphthyl-, phenyl-, benzyl-, phenethyl-, and other arylalkyl isothiocyanates against tumor development in liver, lung, mammary gland, forestomach, and esophagus. Many isothiocyanates and their glucosinolate precursors (β-thioglucoside, *N*-hydroxysulfate) occur naturally and sometimes abundantly in plants consumed by humans, *e.g.*, cruciferous vegetables. Nevertheless, the possible contributions of isothiocyanates and glucosinolates to the well recognized protective effects against cancer of high consumptions of vegetables are unclear. The anticarcinogenic effects of isothiocyanates appear to be mediated by tandem and cooperating mechanisms: (*a*) suppression of carcinogen activation by cytochromes P-450, probably by a combination of down-regulation of enzyme levels and direct inhibition of their catalytic activities, which thereby lower the levels of ultimate carcinogens formed; and (*b*) induction of Phase 2 enzymes such as glutathione transferases and NAD(P)H: quinone reductase, which detoxify any residual electrophilic metabolites generated by Phase 1 enzymes and thereby destroy their ability to damage DNA. Since isothiocyanates block carcinogenesis by dual mechanisms and are already present in substantial quantities in human diets, these agents are ideal candidates for the development of effective chemoprotection of humans against cancer.

Introduction

Organic isothiocyanates (R—N═C═S), also known as mustard oils, are widely distributed in plants, many of which are consumed by humans. They are responsible for the pungent and acrid flavor and odor of condiments such as mustard and horseradish and the familiar biting taste that develops when some cruciferous vegetables are eaten. The designation "mustard oil" originates from the flavor of mustard seeds which is largely due to the presence of abundant quantities of allyl isothiocyanate (CH_2═CH—CH_2—NCS). In plants, isothiocyanates are invariably accompanied by usually much larger quantities of their cognate glucosinolates (β-thioglucoside, *N*-hydroxysulfate). In addition to their characteristic flavors and odors, isothiocyanates have a variety of other pharmacological and toxic activities. These include: goitrogenic activity; antibacterial, antifungal, and antiprotozoal actions; the ability to attract or repel insects; cytotoxicity; the induction of chromosome abnormalities and neoplasia; and the blocking of chemical carcinogenesis. The interesting early history of the chemistry and biology of these compounds has been reviewed by Challenger (1). Several more recent encyclopedic and scholarly reviews of the chemistry, distribution in plants, biosynthesis, and biological properties of glucosinolates and isothiocyanates are available (2–4).

Isothiocyanates arise in plants as a result of enzymatic cleavage of glucosinolates by myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which is released when plant cells are injured. Myrosinase promotes the hydrolysis of glucosinolates and intramolecular (Lossen) rearrangement of intermediates to yield isothiocyanates, hydrogen sulfate, and glucose as the major products (Fig. 1).

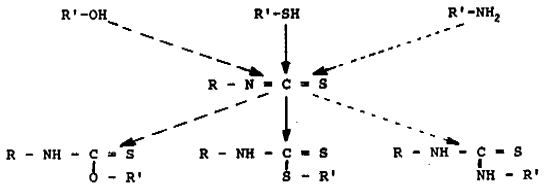
As shown in Fig. 1, a number of other products may also arise from the hydrolysis of glucosinolates by myrosinase. The quantities and nature of these products are variable and appear to be controlled by the chemical nature of the glucosinolate, the pH, and the origin and multiplicity of the myrosinases. A full discussion of the formation of these products is provided by Fenwick *et al.* (2).

Our interest in isothiocyanates stemmed from the observations that several members of this family of substances could block the toxic and neoplastic effects of a wide variety of chemical carcinogens (6). Furthermore, many isothiocyanates are monofunctional inducers of Phase 2 enzymes (7, 8), a property that is associated with protection against chemical carcinogenesis (9). Recently, a very potent isothiocyanate inducer (sulforaphane) has been isolated from broccoli (10). These observations point to the potential importance of isothiocyanates as chemoprotectors against cancer in humans.

This review focuses on the metabolism of isothiocyanates and on the mechanisms of their anticarcinogenic effects. We discuss only experiments in which pure isothiocyanates have been used and omit corroborating information obtained with plants and their extracts, because of the presence of other potentially confounding substances.

Chemical Properties and Metabolism of Isothiocyanates

Chemical Reactivity and Spectroscopic Properties. The highly electrophilic central carbon atom of the —N═C═S group reacts rapidly, and under mild conditions with oxygen-, sulfur-, or nitrogen-centered nucleophiles to give rise to carbamates, thiocarbamates, or



thiourea derivatives, respectively.

These reaction products have been useful for the spectroscopic identification and characterization of isolated isothiocyanates. The —NCS group of isothiocyanates absorbs UV light with low intensity near 240 nm (a_m about 1000 M^{-1} cm^{-1}). Reactions of isothiocyanates with monothiols at pH 7–9 yield thiocarbamates (carbamate thioesters) that show markedly enhanced UV absorption intensity (a_m 10,000 M^{-1} cm^{-1}) and a characteristic maximum at 270 nm with a broad shoulder near 250 nm (11–13). Zhang *et al.* (13) have reported recently that isothiocyanates react with vicinal dithiols, such as 2,3-dimercaptopropanol or ethane-1,2-dithiol, to give rise initially to the expected monothiocarbamates. However, upon further incubation these primary products undergo attack by the free thiol group on the electrophilic carbon, resulting in a cyclization reaction with release of the free amine (13, 14). Thus the reaction of any R-NCS with ethane-1,2-dithiol, 2,3-dimercaptopropanol, and 1,2-benzenedithiol gives rise to ethylene trithiocarbonate (1,3-dithiolane-2-thione, λ_{max} 316 nm; a_m 16,500 M^{-1} cm^{-1}), 4-hydroxymethyl-1,3-dithiolane-2-thione (λ_{max} 316 nm; a_m 16,400 M^{-1} cm^{-1}), and 1,3-benzodithiole-2-thione (λ_{max} 363 nm; a_m 22,500 M^{-1} cm^{-1}), respectively. Based on the

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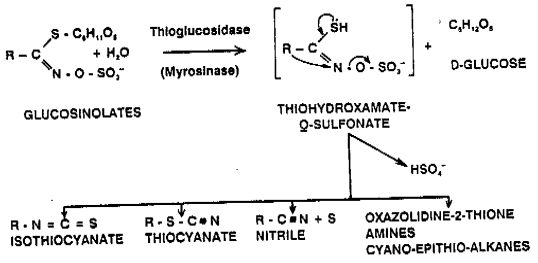

Fig. 1. Hydrolysis of glucosinolates by myrosinase and formation of isothiocyanates and other products [from Pessina *et al.* (5)].

favorable spectroscopic properties of 1,3-benzodithiole-2-thione, a general method for the sensitive, specific, and quantitative analysis of isothiocyanates was developed (13).

Metabolism of Isothiocyanates *in Vivo*. Whereas the nonenzymatic reaction of GSH[2] with benzyl-NCS is rapid, this reaction is also catalyzed by rat liver cytosols, presumably promoted by the glutathione transferases present in these preparations (15). The principal metabolic products of isothiocyanates administered p.o. to mammals are the corresponding thiocarbamates. When six male volunteers were fed about 100 μmol of benzyl-NCS, 53.7% of the dose administered was excreted in the urine as *N*-acetyl-*S*-(*N*-benzylthiocarbamoyl)-L-cysteine, a mercapturic acid, in the first 12 h. No other metabolites were detected, and the excretion appeared to be complete in this period (16). Similar results were observed in two men and two women who were fed watercress, a rich source of phenethyl-NCS and its glucosinolate (gluconasturtiin), which is hydrolyzed by the myrosinase present in this plant (17). When 30- or 57-g portions of fresh watercress (containing 0.72 mg of gluconasturtiin/g) were consumed by each individual, 30 to 67% of the phenethyl-NCS was excreted as *N*-acetylcysteine derivative in the urine in 24 h, and the majority of this conjugate was recovered within the initial 8 h (17). This experiment does not provide unequivocal evidence for the hydrolysis of the glucosinolate to isothiocyanate in the body, since no information on the content of free phenethyl-NCS in the watercress was provided. Furthermore, it is not clear whether breakdown of the glucosinolate to isothiocyanate resulted from the myrosinase activity of the watercress or from enzymes present in the test subjects (either in the host tissues or the microbial flora of the gastrointestinal tract). We are not aware of any experimental information on the ability of human subjects to hydrolyze glucosinolates to isothiocyanates.

Rats, mice, guinea pigs, and rabbits likewise converted isothiocyanates efficiently to *N*-acetylcysteine conjugates, but other metabolites, including cyclic mercaptopyruvate derivatives (not detected in humans) were also formed (17–20). However, when phenyl-, α-naphthyl-, β-naphthyl, or *tert*-butyl isothiocyanate were given to rats, no cysteine conjugates were detected (15, 16, 21).

The conversion of isothiocyanates to their *N*-acetylcysteine derivatives (mercapturic acids) proceeds by the conventional route of conjugation with glutathione presumably promoted by glutathione transferases. The resulting conjugates are then hydrolyzed to the cysteine derivatives and acetylated. Although this pathway has been generally regarded as a detoxication mechanism, recent attention has been directed to the role of GSH and cysteine conjugates (thiocarbamates) as a means of transporting isothiocyanates to peripheral organs where they can undergo cleavage and contribute to toxicity (22, 23) or induce enzymes that protect against toxicity (see below).

Isothiocyanates as Protectors against Chemical Carcinogenesis

The capacity of organic isothiocyanates to block chemical carcinogenesis was recognized 30 years ago (24, 25). Interest in naphthyl-NCS arose from its ability to produce profound proliferative damage in the liver. In long-term feeding experiments α-naphthyl-NCS significantly reduced (in a dose-dependent manner) the formation of liver tumors by 3′-methyl-4-dimethylaminoazobenzene, ethionine, and *N*-2-fluorenylacetamide in male Wistar rats. Furthermore, animals fed α-naphthyl-NCS did not develop ear duct carcinomas and leukemia, unlike rats receiving the *N*-2-fluorenylacetamide alone (24, 25). Lacassagne *et al.* (26) confirmed and extended these findings by demonstrating that not only dietary α-naphthyl-NCS but also β-naphthyl-NCS (which does not cause marked proliferative changes in the liver) blocked hepatic tumor formation in rats fed 4-dimethylaminoazobenzene. Both isothiocyanates produced profound effects on hepatic enzymes that metabolize xenobiotics (27, 28).

These findings laid the groundwork for many subsequent studies on the tumor blocking activities of isothiocyanates, which were administered usually for only short time periods (Table 1).

Mammary Tumors. In the single dose 9,10-dimethyl-1,2-benzanthracene (DMBA)-induced mammary tumor model in female Sprague-Dawley rats, single doses of phenyl-NCS, phenethyl-NCS, and benzyl-NCS markedly reduced the incidence and multiplicity of mammary tumors. In a detailed experiment Wattenberg (29) showed that the timing of administration of the anticarcinogen with respect to the DMBA was very important. Thus, when benzyl-NCS was administered by gavage 2 h prior to the single dose of carcinogen, the average number of rats bearing tumors was reduced from 77% to 8%, and the number of tumors per animal dropped from 1.6 to 0.08. Administration of the isothiocyanate 4 h prior to the carcinogen was somewhat less effective; but if the isothiocyanate was given 24 h before or 4 h after the DMBA, the tumor blocking effect was largely abolished. In other experiments with the same rat tumor model, Wattenberg (30) observed that dietary benzyl isothiocyanate fed for the entire experimental period, beginning 1 week after the DMBA dose, was also highly effective in reducing mammary tumor incidence and multiplicity.

Forestomach and Lung Tumors. Administration of benzyl-NCS (1 mg) to female A/J mice by gavage 15 min prior to each exposure to dimethylnitrosamine (20 mg/kg; once weekly for 8 weeks) reduced forestomach tumor incidence and multiplicity but had no effect on pulmonary adenoma formation. In contrast, administration of benzyl-NCS (1 or 2.5 mg, 15 min before carcinogen) reduced, in a dose-dependent manner, both pulmonary adenomas and forestomach tumors evoked by benzo(*a*)pyrene (3 doses of 2 mg each, given p.o. at 2-week intervals) (31). Benzyl-NCS also suppressed the formation of forestomach tumors in ICR/Ha mice (38).

The effects of isothiocyanates on lung tumors resulting from administration of the potent tobacco-derived nitrosamine carcinogen NNK have been studied intensively and reviewed recently by Chung (39). In male F344 rats, phenethyl-NCS was fed for 1 week prior to and during the course of treatment with NNK (1.76 mg/kg s.c., 3 times weekly for 21 weeks). At the termination of the experiment (104 weeks), the pulmonary tumor incidence (adenomas and carcinomas) was reduced from 80% to 43% by the isothiocyanate treatment, which also inhibited the methylation and pyridyloxobutylation of lung DNA (32).

In shorter term experiments, female A/J mice received 5 μmol of various phenyl-$(CH_2)_n$-NCS (n = 0–6) daily by gavage for 4 days.

[2] The abbreviations and definitions used are: GSH, glutathione; QR, quinone reductase [NAD(P)H:(quinone acceptor) oxidoreductase; EC 1.6.99.2]; GST, glutathione transferases (EC 2.5.1.18); Phase 1 enzymes (principally cytochromes P-450) functionalize xenobiotics largely by oxidative or reductive reactions; Phase 2 enzymes conjugate functionalized compounds with endogenous ligands (e.g., GSH, glucuronic acid). QR is classified as a Phase 2 enzyme. Monofunctional inducers elevate Phase 2 enzymes without intervention of the *Ah* (Aryl hydrocarbon) receptor and do not significantly affect Phase 1 enzyme activities; DMBA, 9,10-dimethyl-1,2-benzanthracene; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; i.g., intragastrically.

Table 1 *Protection against chemical carcinogenesis by aryl and arylalkyl isothiocyanates in rats and mice*

Carcinogen	Species (sex/strain)	Tumor target organ	Protective isothiocyanate	Ref.
3'-Me-DAB[a]	Rat (♂, Wistar)	Liver	α-Naphthyl-	Sasaki (25)
Ethionine	Rat (♂, S-D)	Liver	α-Naphthyl-	Sidransky *et al.* (24)
AAF	Rat (♂, Wistar)	Liver, leukemia, ear duct	α-Naphthyl-	Sidransky *et al.* (24)
DAB	Rat (♂, Wistar)	Liver	α- and β-naphthyl-	Lacassagne *et al.* (26)
DMBA	Rat (♀, S-D)	Mammary gland	Phenyl-, benzyl-, phenethyl-	Wattenberg (29, 30)
DMBA	Mouse (♀, ICR/Ha)	Lung, forestomach	Benzyl-, phenethyl-	Wattenberg (29)
BP	Mouse (♀, ICR/Ha)	Forestomach	Benzyl-	Wattenberg (29)
DEN	Mouse (♀, A/J)	Forestomach	Benzyl-	Wattenberg (31)
BP	Mouse (♀, A/J)	Lung, forestomach	Benzyl-	Wattenberg (31)
NNK	Rat (♂, F344)	Lung	Phenethyl-	Morse *et al.* (32)
NNK	Mouse (♀, A/J)	Lung	Phenyl- $(CH_2)_n$-(n = 0–6)	Morse *et al.* (33–35)
NBMA	Rat (♂, F344)	Esophagus	Phenethyl-	Stoner *et al.* (36, 37)

[a] 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; DAB, 4-dimethylaminoazobenzene; AAF, *N*-2-fluorenylacetamide; BP, benzo(*a*)pyrene; DEN, diethylnitrosamine; NBMA, *N*-nitrosobenzylmethylamine.

Two h after the final treatment with isothiocyanates, a single dose of NNK (10 μmol by i.p. injection) was administered and pulmonary tumors were quantitated 16 weeks later. Phenyl-NCS and benzyl-NCS were inactive, but phenethyl-NCS (n = 2) and isothiocyanates with n = 3–6 produced marked reductions in lung tumor formation; the inhibition was progressively more pronounced as the methylene chain was lengthened (33, 34). Thus phenylhexyl-NCS (5 μmol daily for 4 days) reduced pulmonary tumor multiplicity from 7.9 to essentially zero. DNA methylation, as measured by O^6-methylguanine formation, was likewise blocked by these arylalkyl isothiocyanates, again more markedly as the methylene chain of the isothiocyanates was made longer (34).

If the administration of benzyl-NCS or phenethyl-NCS (1–3 μmol/g of diet) was delayed for 1 week after treatment with NNK and then continued to the end of the experiment at 16 weeks, no effect on pulmonary tumor formation was observed at nontoxic doses of the isothiocyanates (35).

Tumors of the Esophagus. Stoner *et al.* (36) have reviewed their studies on the inhibition by phenethyl-NCS of esophageal tumor production in the rat by the asymmetrical nitrosamine, *N*-nitrosobenzylmethylamine. The importance of these studies derives from the belief that *N*-nitroso compounds and their precursors are probably causative factors for esophageal cancers in some high incidence regions (37). Male F344 rats treated with *N*-nitrosobenzylmethylamine, (0.5 mg/kg s.c. once per week for 15 weeks) developed 100% esophageal tumors at the end of the 25-week assay period, and the tumor multiplicity was 11.5/animal. In experimental groups also fed phenethyl-NCS (3 μmol/g of diet), tumor incidence was only 13% and average tumor multiplicity was negligible (0.1 ± 0.3/animal). At a higher dose of phenethyl-NCS (6 μmol/g of diet) no tumors were observed (37). Phenethyl-NCS treatment blocked the formation of both preneoplastic and neoplastic lesions in the esophagus; even at the lower dose of the isothiocyanate, there was complete inhibition of the appearance of the more advanced (papilloma and carcinoma) lesions (37).

Parallel measurements of the effects of phenethyl-NCS on the metabolism of [^{3}H]nitrosobenzylmethylamine in rat esophageal explants demonstrated concentration-dependent inhibition of both metabolism of the carcinogen and methylation of DNA, as indicated by decreased formation of N^7-methylguanine and O^6-methylguanine.

Tumor-blocking Effects of Glucosinolates. A few experiments have examined the chemoprotective effects of glucosinolates, which are often present in plants in much higher concentrations than their isothiocyanate hydrolysis products. The most clear-cut results have been obtained with the DMBA-induced mammary tumors of the Sprague-Dawley rat. Administration of large single doses of glucobrassicin (indolylmethyl glucosinolate) or of glucotropaeolin (benzyl glucosinolate) 4 h prior to the carcinogen substantially reduced both the incidence (from 75% to 25–38%) and the multiplicity (from 1.25 to 0.50–0.69 tumor/rat) of mammary tumors. Administration of these glucosinolates or of glucosinalbin (4-hydroxybenzyl glucosinolate) produced some reduction in the multiplicity, but usually not the incidence, of forestomach tumors and pulmonary adenomas of mice treated with benzo(*a*)pyrene (40). The interpretation of these experiments is complicated. It is not known to what extent these glucosinolates are degraded in the rodent bodies or to what extent the glucosinolates or their degradation products are responsible for the tumor-blocking effects. If the glucosinolates are hydrolyzed, it is not known whether endogenous or microbial enzymes are responsible and what products are formed. These issues are especially complicated for glucobrassicin, since three known degradation products of this glucosinolate (indole-3-acetonitrile, 3,3'-diindolylmethane, and indole-3-carbinol) are anticarcinogens and inducers of cytochrome P-450 (41, 42). A further potentially confounding effect is the conversion of indole-3-carbinol under mildly acid conditions (such as prevail in the stomach) to cyclic derivatives that bind at very low concentrations to the *Ah* receptor and thereby become very potent inducers of cytochrome P450IA1 (43).

Mechanisms: Effects of Isothiocyanates on Carcinogen Metabolism

Understanding of the mechanisms of the chemoprotective effects of isothiocyanates is of great importance not only because these substances block the formation of a wide variety of carcinogen-induced tumors in rodents, but also because isothiocyanates and their glucosinolate precursors are widespread in human dietary plants and are consumed in substantial quantities. To what extent these substances contribute to the well-recognized protective effects of vegetables against cancer is unclear (44). The only plausible mechanisms proposed for the anticarcinogenic effects of isothiocyanates implicate modulation of carcinogen metabolism, both depression of activation of carcinogens and acceleration of their disposal. Evidence for these conclusions is based on measurements of: (*a*) carcinogen-DNA adduct formation and the accompanying nucleotide modifications; (*b*)

Table 2 *Induction of Phase 2 enzymes by isothiocyanates in rodent tissues*

Enzyme	R-NCS	Species (sex/strain)	Tissue	Ratio of enzyme specific activities (treated/ control)	Ref.
GST	Benzyl-NCS	Mouse (female; ICR/Ha)	Esophagus	2.08–2.59	Sparnins *et al.* (38, 49, 50)
			Forestomach	2.46	
			Liver	3.20	
			Small bowel	4.38–9.36	
GST	Benzyl-NCS	Mouse (female; ICR/Ha)	Forestomach	3.43–4.29	Benson and Barretto (51)
			Liver	3.0–3.5	Benson *et al.* (52)
			Lung	1.63–1.73	
			Kidney	1.35–1.37	
			Small bowel	5.27–8.31	
			Colon	1.25	
GST	Allyl-NCS	Rat (male; F344)	Liver	1.26–2.25	Bogaards *et al.* (53)
			Small bowel	3.07–3.93	
GST	Phenethyl-NCS	Rat (male; F344)	Liver	1.3	Guo *et al.* (47)
GST	Phenylbutyl-NCS	Rat (male; F344)	Liver	1.35	Guo *et al.* (57)
	Phenylhexyl-NCS		Liver	1.37	
GST	Sulforaphane	Mouse (female; CD-1)	Forestomach	1.08 – 3.00	Zhang *et al.* (10)
	Erucin		Glandular stomach		
	Erysolin		Liver		
			Small bowel		
			Colon		
QR	Benzyl-NCS	Mouse (female; CD-1)	Forestomach	2.57	Benson and Barretto (51)
			Liver	2.05	Benson *et al.* (52)
			Lung	2.82	
			Kidney	2.27	
			Small bowel	4.78	
			Colon	1.80	
			Bladder	2.41	
QR	Phenethyl-NCS	Rat (male; F344)	Liver	5.1	Guo *et al.* (47)
			Lung	~1	
			Nasal mucosa	~1	
QR	Phenylbutyl-NCS	Rat (male; F344)	Liver	1.44	Guo *et al.* (57)
	Phenylhexyl-NCS		Liver	1.63	
QR	α-Naphthyl-NCS	Rat (male; F344)	Liver	2.8–4.4	Leonard *et al.* (27, 28)
	β-Naphthyl-NCS		Liver	2.23	
QR	Sulforaphane	Mouse (female; CD-1)	Forestomach	1.05–3.10	Zhang *et al.* (10)
	Erucin		Glandular stomach		
	Erysolin		Liver		
			Small bowel		
			Colon		

rates of activation of carcinogens and levels of Phase 1 enzymes; and (*c*) activities of Phase 2 enzymes and levels of GSH.

Reactions of Carcinogens with DNA. There is a striking parallel between the inhibitory effects of various arylalkyl isothiocyanates on lung tumor formation by NNK, the potent carcinogenic nitrosamine of tobacco, and the ability of these isothiocyanates to block O^6-methylguanine formation in lungs of rats and mice. The very systematic studies by Chung, Hecht and their colleagues should be consulted for details [Refs. 32, 39, and 45; reviewed by Chung (46)]. These workers and others (46) have also studied the metabolic activation of NNK by lung tissue and microsomes and have concluded that the chemoprotective isothiocyanates act principally on the enzymes involved in the metabolic activation of NNK. By the use of inhibitory antibodies specific for cytochromes P-450, Guo *et al.* (46) identified cytochromes P450IIB1 and 2 but not P450IA1 or P450IIE1 as important participants in the activation process.

Similar experiments by Stoner *et al.* (36, 37) have established that the inhibitory effects of phenethyl-NCS on the production of esophageal tumors by *N*-nitrosobenzylmethylamine in rats also paralleled inhibition of the binding of the carcinogen to DNA and the formation of N^7-methylguanine and O^6-methylguanine.

Regulation of Phase 1 Enzymes by Isothiocyanates. Administration of isothiocyanates to rodents produced either increases or decreases of microsomal cytochrome P-450 content and the activities of several cytochrome P-450-dependent monooxygenases. The effects appear to depend on experimental conditions: the nature of the isothiocyanate; the treatment regimen; the target tissue examined; and the specific monooxygenase measured.

The most dramatic increases have been reported with indole-3-carbinol and 3,3′-diindolylmethane and to a lesser degree with indole-3-acetonitrile. These indoles are hydrolytic rearrangement products of glucobrassicin (indolylmethyl glucosinolate). Single i.g. administrations of 10–100 μmol of these indoles to female Sprague-Dawley rats raised the aryl hydrocarbon hydroxylase activities of homogenates of liver and small intestine as much as 25-fold. Clearly these effects cannot be directly attributed to the actions of isothiocyanates (41). As already mentioned,

recent experiments (43) have shown that indole-3-carbinol is readily converted both *in vitro* and *in vivo*, under mildly acid conditions to polymeric indoles that bind with very high affinity to the *Ah* receptor, and thereby enhance transcription of cytochrome P450IA1.

Single i.g. doses (100 μmol) of phenethyl-NCS to male F344 rats caused marked increases of liver microsomal pentoxyresorufin *O*-dealkylase activity (10-fold) and the content of cytochrome P450IIB1 protein (6.6-fold) (47). In contrast, cytochrome P-450 content, ethoxyresorufin *O*-dealkylase, and erythromycin *N*-demethylase activities all fell moderately during the first 12–18 h and then recovered by the end of the experiment at 48 h (47). Although these effects appear to be somewhat contradictory, the above-described treatment with phenethyl-NCS depressed the formation of reactive metabolites of NNK by lung (and to a lesser degree by liver) microsomes. The principal effect of phenethyl-NCS appears to be depression of the α-hydroxylation of NNK which is critical for DNA adduct formation. Furthermore, the direct addition of a number of isothiocyanates to microsomes of rat and mouse lungs and rat nasal mucosa potently depressed the metabolic activation reactions of NNK. Thus, 50% inhibition was achieved by phenylethyl-NCS at 120–200 nM, by phenylbutyl-NCS at 30–75 nM, and by phenylhexyl-NCS at 15–90 nM depending on the specific metabolites. The K_i values for the inhibition of NNK metabolism by phenylhexyl-NCS were very low (10.9–16.8 nM) for various reactions (57).

Straightforward results were obtained in experiments in which 100–300 μmol daily i.g. doses of allyl-NCS were given to male outbred rats for 3 days. The hepatic aminopyrine *N*-demethylase, *p*-nitroanisole *O*-demethylase, and aniline *p*-hydroxylase activities were all decreased in a dose-dependent manner to as low as 31–46% of control values (48).

Although both α- and β-naphthyl-NCS block neoplasia in the liver (see above), dietary administration of these compounds to male F344 rats for 2–4 weeks had opposite effects on cytochrome P-450 content and monooxygenase activities of liver microsomes. Whereas the α-naphthyl-NCS depressed total P-450 content, as well as ethoxycoumarin *O*-deethylase and benzphetamine *O*-demethylase activities, the β isomer had the opposite effects (27, 28). For instance, dietary β-naphthyl-NCS (5.4 μmol/g diet for 28 days) raised microsomal cytochrome P-450 content, ethoxycoumarin *O*-deethylase, and benzphetamine *N*-demethylase 1.6-, 1.74-, and 2.2-fold, respectively (28).

In Hepa 1c1c7 murine hepatoma cells in culture, benzyl-NCS had no effect on aryl hydrocarbon hydroxylase activity (8).

Although the effects of isothiocyanates on Phase 1 enzymes appear to vary greatly with experimental conditions and the activity measured, the conclusion that these compounds depress the activational metabolism of two carcinogens, NNK and *N*-nitrosobenzylmethylamine, appears to be firmly established.

Regulation of Phase 2 Enzymes and Glutathione Levels by Isothiocyanates

In contrast to the complex effects of isothiocyanates on Phase 1 enzymes, the effect of these agents on Phase 2 enzymes of rodent tissues is quite straightforward. Table 2 summarizes the changes in specific activities of GST and QR in the cytosols of several organs of mice and rats treated with benzyl-NCS, phenethyl-NCS, allyl-NCS, α-naphthyl-NCS, β-naphthyl-NCS, or sulforaphane [1-isothiocyanato-(4*R*)-(methylsulfinyl)butane]. The compounds were given either in the diet (3–34 μmol/g diet) for 5–28 days or by i.g. administration (5–100 μmol in single or several daily doses). The organs examined included: liver; esophagus; forestomach; glandular stomach; small bowel; colon; lung; kidney; and bladder. Inductions of GST and QR, expressed as ratios of specific activities of tissues obtained from treated and control animals varied from 1.2 to 9.4 and were mostly in the 2–4-fold elevation range. Phase 2 enzyme induction, as represented by elevations of GST and QR in various tissues, appears to be a constant property of a variety of isothiocyanates. Benzyl-NCS also raised GSH levels in esophagus and small bowel (but not forestomach) of mice by 63–75% (Table 3; Ref. 38).

Table 3 *Effects of benzyl isothiocyanate on glutathione levels in female ICR/Ha mouse tissues*

Tissue	Ratio of GSH concentrations (treated/control)	Ref.
Esophagus	1.63–1.75	Sparnins *et al.* (49)
Forestomach	0.77	Sparnins and Wattenberg (38)
Small bowel	1.64–1.66	Sparnins *et al.* (49)

Table 4 *Induction of quinone reductase in Hepa 1c1c7 murine hepatoma cells by isothiocyanates*[a]

Isothiocyanate	Concentration required to double QR (μM)
Sulforaphane [$CH_3SO(CH_2)_4NCS$]	0.2
N-Hexyl-NCS	15
Benzyl-NCS	1.9
Cyclooctyl-NCS	10
Cyclohexyl-NCS	50

[a] From Prestera *et al.* (55).

In common with many chemically unrelated chemoprotective compounds, the administration of isothiocyanates to rodents evokes a generalized "electrophile counterattack" response, characterized by the induction of Phase 2 enzymes and increases in tissue GSH levels (54, 55).

Much has been learned about the chemical structure and mechanism of induction of chemoprotective compounds by the measurement of QR activity in Hepa 1c1c7 murine hepatoma cells in culture (7), and especially in cells grown in 96-well microtiter plates (56). Elevation of QR in this cell line accurately predicts induction of both QR and GST in animal tissues. This system played a critical role in the identification of sulforaphane as the major Phase 2 enzyme inducer in SAGA broccoli (10). Extensive information is now available on the inducer potency of a wide variety of isothiocyanates in Hepa cells. Some illustrative inducer concentrations required in the assay system to double the specific activity of QR in Hepa 1c1c7 murine hepatoma cells are given in Table 4.

In an effort to understand the basis for the high inducer potency of sulforaphane, a large number of bifunctional isothiocyanates were recently synthesized and evaluated as inducers (58). It was found that among these analogues, the methylsulfinyl group (CH_3SO—) of sulforaphane could be replaced by either CH_3CO— or CH_3SO_2— groups without significant effect on inducer potency. For optimal potency, these functional groups had to be separated from the —NCS function by 3 or 4 carbon atoms. In some of the analogues, the link separating the two functions was flexible whereas in others it was relatively rigid. Certain bifunctional norbornyl-NCS analogues were potent inducers of QR in murine hepatoma cells and inducers of QR and GST in mouse tissues.

Since there is much persuasive evidence that induction of Phase 2 enzymes plays a major role in the chemoprotective effects of many different classes of compounds (9), it appears very likely that isothiocyanates also exert at least a part of their protective actions through this mechanism. Isothiocyanates are potent electrophiles like most other inducers of Phase 2 enzymes (7, 54, 55). Isothiocyanates resemble other monofunctional enzyme inducers in that they stimulate transcription of Phase 2 enzymes via a common AP-1-like enhancer element present in the upstream regulatory regions of certain GST and QR genes (54, 55).

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THE ROLE OF ENZYME INDUCTION IN PROTECTION AGAINST CARCINOGENESIS*

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I. INTRODUCTION AND BACKGROUND

The notion that protection against the neoplastic effects of carcinogens can be achieved by administration of chemical agents is by no means new. Twenty-five years ago, in a review entitled "Chemo-prophylaxis of Carcinogenesis," Wattenberg[1] recorded experiments going back to 1929, in which chemical induction of tumors in rodents had been blocked by a variety of chemicals, including polycyclic aromatic hydrocarbons, phenothiazines, actinomycin D, α,β-unsaturated dicarboxylic acids and anhydrides, dichlorodiethyl sulfide (mustard gas), metal ions, 2,3-dimercaptopropanol, 4-nitroquinoline *N*-oxide, and others. Although mouse skin was the experimental model for most of these studies, protection was also observed against systemic tumor formation in liver, mammary gland, and connective tissue, thereby indicating that protection did not arise solely from a local interaction between carcinogen and protector. One striking feature of these observations was that the protective agents were often carcinogens themselves and that under appropriate experimental conditions established carcinogens could act as protectors. Indeed, prior treatment with low doses of a carcinogen protected against higher doses of the same carcinogen.[2]

The striking finding of Richardson and Cunningham[3] that small doses of 3-methylcholanthrene (a potent carcinogen) provided substantial protection against the generation of liver tumors by the azo dye butter yellow (3'-methyl-*N*,*N*,-dimethylaminoazobenzene) was followed many years later by the demonstration of the reverse phenomenon: protection against hydrocarbon (7,12-dimethylbenz(a) anthracene)-induced mammary tumor formation in rats by small doses of the the azo dye, Sudan III.[4]

*This solicited summary of work from our laboratory on chemoprotection against cancer is based on several recent reviews that are referenced in the bibliography. A similar chapter will appear as part of the Proceedings of a Workshop on "Cellular and Molecular Targets for Chemoprevention," sponsored by the National Cancer Institute and held in Charleston, South Carolina, on March 16-19, 1991.

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Insight into the mechanisms responsible for these phenomena was provided by James and Elizabeth Miller and their coworkers,[5,6] who in the course of their analyses of the biochemical basis of chemical carcinogenesis attributed the protective effects to the greatly enhanced rates of oxidative and reductive detoxication of the carcinogenic azo dye (reduction of the azo linkage, ring hydroxylation, and *N*-demethylation) resulting from the induction of enzymes of xenobiotic metabolism. The responsible enzymes are now known as cytochromes P-450 (Phase I enzymes).[7] In retrospect this explanation is probably incomplete because enhancement of metabolic conjugation by Phase II enzymes (see below) undoubtedly also played a role in the protective phenomenon.

Carcinogenesis is a complex and protracted multistage process. Until recently, reduction or avoidance of exposure to carcinogens and early diagnosis were the only hopes of reducing the incidence of cancer or achieving better therapeutic results. It is now evident that the events that occur between exposure to carcinogens and the ultimate development of overt malignancy offer many opportunities for blocking or even reversing the neoplastic process. Our laboratory has focused on developing strategies for protecting cells against the effects of carcinogens by enhancing their metabolic inactivation, thereby promoting their elimination and reducing the opportunities for damaging interactions with cellular macromolecules.[8,9] Several considerations make this an especially appealing target for achieving chemoprotection: (a) protection is implemented at a very early step of the carcinogenic process, before damage to DNA occurs; (b) the enzymology of the metabolic reactions involved in carcinogen activation and inactivation is reasonably well understood; (c) many inducers of enzymes of carcinogen metabolism have been identified and the structures of others can now be predicted; and (d) a number of inducers of detoxication enzymes are relatively nontoxic; some occur naturally in living matter, and others are already minor components of the human diet.

II. ENZYME INDUCTION AND CHEMOPROTECTION

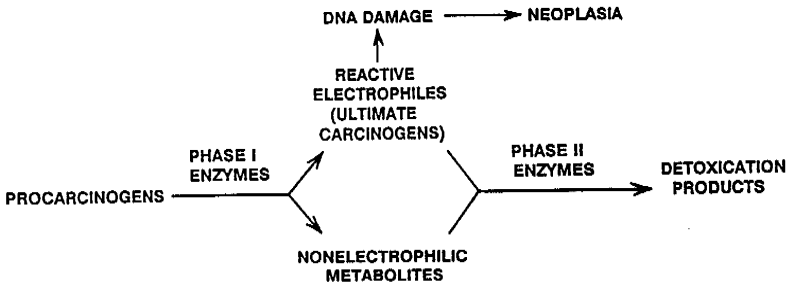
A. METABOLISM OF CARCINOGENS

The critical importance of metabolic activation of chemical carcinogens to their ultimate reactive forms was uncovered by the Millers and their colleagues[10] to whom we owe the central dogma of carcinogenesis: that ultimate carcinogens are electrophilic species that enter into damaging interactions with nucleophilic centers on the bases of DNA and thereby participate in carcinogenesis. These metabolic activations are generally promoted by one or more members of the family of inducible cytochromes P-450 (designated Phase I enzymes), which are membrane-bound hemoproteins that carry out mainly oxidations and reductions. Many (perhaps the majority) of the metabolic reactions catalyzed by cytochromes P-450 do not, however, lead to metabolic activations but to the formation of more water-soluble, nonelectrophilic detoxication products. Prominent among enzymes responsible for activation are cytochrome P-450IA1, involved in conversion of benzo(a)pyrene and other polycyclic aromatics to highly carcinogenic diol epoxides, and cytochrome P-450IA2, which participates in the enhancement of the carcinogenic functions of aromatic amines through their N-hydroxylation. Both of these cytochromes P-450 are inducible by a mechanism that requires the participation of the cytosolic *Ah* (*A*ryl *h*ydrocarbon) receptor. The liganded *Ah* receptor is transported to the nucleus where it binds and activates XRE (Xenobiotic Responsive

Elements) in the upstream regions of these cytochrome P-450 genes.[11] Strong evidence for the importance of the *Ah* receptor in carcinogenesis is provided by the greatly reduced susceptibility to carcinogens of inbred mouse strains that lack or have low levels of this receptor.[12,13]

Protection of cells against the very reactive electrophilic metabolites of carcinogens and other xenobiotics is provided by the high intracellular levels of glutathione, which acts as a noncritical nucleophile scavenger to inactivate these toxic substances. Important defenses against electrophile toxicity are also provided by families of Phase II enzymes that inactivate these electrophiles by conjugation with glutathione (glutathione *S*-transferases), or with glucuronic acid (UDP-glucuronosyltransferases), or by hydrolysis. Another important detoxication enzyme is NAD(P)H:quinone reductase (quinone reductase), which catalyzes the obligatory two-electron reductions of quinones and thereby shields cells against the damaging electrophilicity of quinones and their ability to generate oxidative stress by oxidative cycling.[14] Phase II enzymes also conjugate and otherwise modify the nonelectrophilic products of Phase I enzyme action.

A simplied general scheme for the role of Phase I and Phase II enzymes in carcinogen metabolism is shown below:



The discovery by Wattenberg[15,16] that the phenolic antioxidant food additives, BHA [2(3)-*tert*-butyl-4-hydroxyanisole] and BHT (2,6-di-*tert*-butyl-4-hydroxytoluene), protected rodents against the the toxic and neoplastic effects of a variety of carcinogens provided an important impetus to the development of the field of chemoprotection. Because these phenolic antioxidants were already present in the human diet, and clearly could not be very toxic, the prospects that other nontoxic chemoprotectors would be identified seemed much brighter.

Our analysis of the mechanism of the protective effects of these agents led to the startling observation that administration of phenolic antioxidants (and as subsequently shown, a wide variety of other chemoprotectors) produced marked elevations of glutathione transferases and other Phase II enzymes in many tissues without major effects on cytochromes P-450 (Phase I enzymes).[17-21] These findings suggested strongly that elevation of Phase II enzymes was a critical (and sufficient) condition for achieving chemoprotection.

An obvious corollary of this conclusion was that the balance between Phase I and Phase II enzymes could exercise critical control over the fate of carcinogens and that shifting this balance toward Phase II enzyme predominance could result in protection against electrophile toxicity and carcinogenesis. Subsequent observations from many laboratories have fully corroborated these suggestions.

B. MODULATION OF ENZYMES OF CARCINOGEN METABOLISM AND CONTROL OF CARCINOGENESIS

Since Wattenberg's 1966 review[1] of chemoprotectors, the list of compounds capable of protecting animals against the toxic and neoplastic effects of carcinogens has grown substantially, both in number and chemical diversity.[22,23] Chemoprotectors include not only small doses of polycyclic aromatics (some of which are themselves carcinogens) and azo dyes but also phenolic antioxidants such as BHA and BHT, coumarins, flavonoids, organic isothiocyanates, thiocarbamates (for example, disulfiram, diethyldithiocarbamate, bisethylxanthogen), 1,2-dithiole-3-thiones, indoles, and cinnamates.[22,23] It was difficult to understand how administration of such a bewildering variety of compounds could protect against carcinogenesis. Yet many experiments showed that these chemoprotectors were endowed with a singular and universal property: they were inducers of enzymes of xenobiotic metabolism in many tissues *in vivo* and in a variety of cells in culture. Many lines of evidence suggested that the ability to enhance the metabolic inactivation of carcinogens was largely responsible for the chemoprotective properties of these agents.[8]

More detailed analysis of the enzyme induction patterns evoked by these chemoprotectors led to the recognition that they fell into two quite distinct families, which we have designated *monofunctional* and *bifunctional* inducers.[24,25] Monofunctional inducers elevate the specific activities of Phase II enzymes without producing significant effects on the activities of cytochromes P-450 that are under the regulation of the *Ah* receptor. By contrast, bifunctional inducers are generally large planar aromatic compounds that bind to the *Ah* receptor and elevate the activities of both Phase I and Phase II enzymes. Because elevation of Phase II enzymes appears to be a sufficient condition for achieving protection against carcinogenic electrophiles, and Phase I enzymes can participate in carcinogen activation, chemoprotective strategies should ideally be implemented with monofunctional rather than bifunctional inducers.

C. EXPERIMENTAL SYSTEMS FOR THE STUDY OF MONOFUNCTIONAL INDUCERS

Because of the compelling evidence that Phase II enzyme induction is associated with a chemoprotected state (reduced susceptibility to the toxic and neoplastic effects of chemical carcinogens), we have developed simple experimental systems for identifying novel monofunctional inducers, for measuring their potencies, and for elucidating the molecular events responsible for induction. NAD(P)H:quinone oxidoreductase is a widely distributed FAD-containing flavoprotein that protects cells against the toxicities of quinones.[14] It is coordinately induced with other Phase II enzymes in most tissues. The murine hepatoma cell line designated Hepa 1c1c7 contains inducible quinone reductase that is easily measurable. This cell line therefore provides a reliable system for detecting and for measuring potency of monofunctional as well as bifunctional inducers.[26,27] The utility of this system has been greatly extended by growing Hepa 1c1c7 cells in microtiter plate wells and measuring

directly the specific activities of quinone reductase by means of a computer-coupled microtiter plate reader.[28] Quinone reductase is induced in these cells by all of the chemoprotectors shown in Figure 1.

D. IDENTITY OF CHEMICAL SIGNAL RESPONSIBLE FOR MONOFUNCTIONAL INDUCTION

Monofunctional inducers appear to act independently of any conventional receptor and superficially reveal few, if any, common structural features. Nevertheless a systematic study of the relation of structure to inducer potency led to the conclusion that all monofunctional inducers contain (or acquire by metabolism) electrophilic reaction centers.[9,29] This conclusion was reached in the following manner. When the inducer potencies of a series of alkyl ethers of 1,4-diphenols (designed as analogues of BHA) were examined, unsubstituted *tert*-butylhydroquinone was the most potent inducer and was active in tissues in which the ethers were only feebly active.[30] When these experiments were extended to include not only 1,4-diphenols but also 1,2-diphenols (catechols) and 1,3-diphenols (resorcinols), it was consistently found that only 1,2-diphenols and 1,4-diphenols were inducers, whereas 1,3-diphenols were completely inactive.[27,31] The appropriate orientation of the phenolic hydroxyl groups was absolutely critical, whereas the presence or absence of other ring substituents had only minor effects on inducer potency. Similarly, among phenylenediamines the 1,2- and 1,4-isomers were inducers, but the 1,3-phenylenediamine was not. Since in these two series of compounds the 1,2- and 1,4-isomers are readily oxidized to quinones or quinoneimines whereas the 1,3-isomers cannot undergo such oxidations, we concluded that oxidative lability was an essential requirement for inducer activity.[27] Nevertheless, these experiments did not disclose whether the oxidative process (and for instance the attendant generation of reactive oxygen species) was responsible for the induction of Phase II enzymes, or whether the quinones or quinoneimines themselves provided the inductive signals.

Dissection of the structure of coumarin (an inducer lactone) and related compounds revealed that only the α,β-unsaturated lactone moiety was required for inducer activity, and that indeed α,β-unsaturated ketones were even more potent inducers.[29] These findings suggested that the critical structure responsible for inducer activity was the electrophilic olefin, which was activated by conjugation to a carbonyl or another electron-withdrawing group. The reactivity of such olefins is the basis for the well-known Michael reaction of electrophilic olefins with nucleophiles. This finding explained the inducer activity of oxidizable diphenols and phenylenediamines because their quinone and quinoneimine oxidation products, respectively, are excellent Michael reaction acceptors.

These generalizations not only explained the activities of many known inducers but also led to the prediction of inducer activity among acrylates, crotonates, cinnamates, fumarates, maleates, vinyl ketones, vinyl sulfones, and others.[9,29,32,33] Furthermore, the inducer potency of these compounds generally correlated well with their reactivity as Michael reaction acceptors. For instance, nitro-olefins such as 1-nitro-1-cyclohexene were very potent inducers, as expected on the basis of the highly potent electron-withdrawing effect of the nitro group.

In light of the natural occurrence of fumarates in animal tissues and the generally recognized low toxicity of fumarate, which is used as a food additive, we have examined the

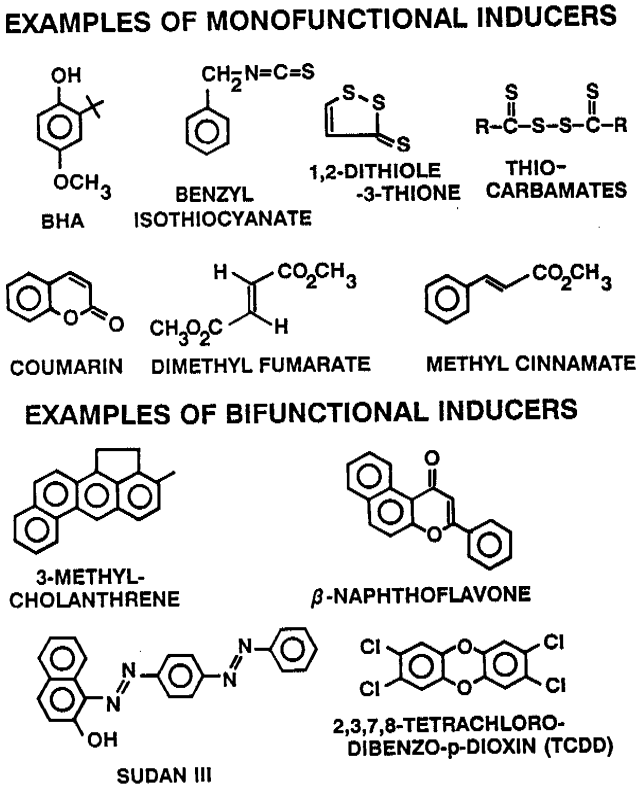


FIGURE 1. *Examples of monofunctional inducers that elevate Phase II enzymes selectively and bifunctional inducers that raise Phase I and Phase II enzymes. For dithiocarbamates: disulfiram (R = $(C_2H_5)_2N$); bisethylxanthogen (R = C_2H_5O-).*

inducer activity of dimethyl fumarate in rodent tissues. Dietary administration of this compound to mice and rats raised the specific activities of quinone reductase and glutathione transferase in many tissues two- to elevenfold.[32] These findings have encouraged us to suggest that dimethyl fumarate be examined for its protective activity against carcinogenesis. This suggestion gains validity from the finding that fumaric acid itself is a plant product and is endowed with a variety of pharmacological properties, including the ability to block chemical carcinogenesis and other forms of electrophile toxicity (see ref. 32 and references therein).

E. INDUCERS ARE SUBSTRATES FOR GLUTATHIONE TRANSFERASES

The striking similarity of the requirements for inducer activity and for substrates of glutathione transferases became immediately apparent as the structure/activity relationship was developed.[33] Thus in an extensive survey of glutathione transferase substrates, Chasseaud[34] described many of the very same compounds that were subsequently shown to be inducers of Phase II enzymes. A systematic survey of the ability of a broad range of glutathione transferase substrates to induce quinone reductase activity in Hepa 1c1c7 cells revealed virtual identity of structural requirements. Thus, for instance, the following transferase substrates were all inducers: 1-chloro-2,4-dinitrobenzene, methyl iodide, 1,4-dichloro-2-nitrobenzene, ethacrynic acid, cumene hydroperoxide, and *tert*-butyl hydroperoxide.[33] However, it is not clear whether this correlation of requirements for inducer and substrate activity occurs because both processes depend on the presence of electrophilic centers, or whether glutathione transferases participate in the transmission of the electrophilic inducer signal to activate the enhanced transcription of Phase II enzymes.

III. CONCLUSIONS AND SUMMARY

The susceptibility of biological systems to carcinogens is controlled at least in part by the balance between Phase I enzyme systems (cytochromes P-450), which activate carcinogens to their ultimate and highly reactive electrophilic forms, and Phase II enzymes (for example, glutathione transferases, epoxide hydrolases, and NAD(P)H:quinone reductase), which convert these carcinogens to less toxic products. Both Phase I and Phase II enzymes are induced by a variety of chemical agents. Such inducers fall into two families: bifunctional inducers (mostly large planar aromatics) that induce both Phase I and Phase II enzymes, and monofunctional inducers that elevate Phase II enzymes selectively. Protection can be achieved by raising the levels of Phase II enzymes only, and because Phase I enzymes are involved in the activation of carcinogens, practical strategies for chemoprotection ideally should concentrate on monofunctional inducers. Monofunctional inducers contain, or acquire by metabolism, electrophilic centers, and many are Michael reaction acceptors. It is therefore quite easy to design or to identify monofunctional inducers, and many inducers of this type are already present in the human diet. The feasibility of modifying the human diet to increase the consumption of foods containing monofunctional inducers needs consideration as a strategy for chemoprotection.

IV. ACKNOWLEDGMENTS

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